AD_____

AWARD NUMBER: W81XWH-04-1-0010

TITLE: Treatment of Prostate Cancer with a DBP-MAF-Vitamin D Complex to Target Angiogenesis and Tumorigenesis

PRINCIPAL INVESTIGATOR: Michael W. Fannon, Ph.D.

CONTRACTING ORGANIZATION: University of Kentucky Research Foundation
                           Lexington, Kentucky   40506-0057

REPORT DATE: February2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

REPORT DOCUMENTATION PAGE

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-02-2006	Annual	1 Feb 2005 – 31 Jan 2006

4. TITLE AND SUBTITLE

Treatment of Prostate Cancer with a DBP-MAF-Vitamin D Complex to Target Angiogenesis and Tumorigenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0010

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Michael W. Fannon, Ph.D.

E-Mail: michael.fannon@uky.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Kentucky Research Foundation
Lexington, Kentucky 40506-0057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study is investigating the potential combination therapy of DBP-maf and vitamin D on prostate tumors using human models of prostate cancer in a mouse model. Initial results have demonstrated that DBP-maf has both anti-angiogenic and anti-tumorigenic properties in vitro. Direct effects on tumor cell proliferation were observed in the parental but not metastatic form of LnCaP cells. This finding could be significant in characterizing differential responses of primary and metastatic tumor sites to therapies. These data will be integrated in the development of initial conditions for our in vivo tumor models. The use of a DBP-maf peptide was also tested in in vitro assays. This work was undertaken based on a published report that the peptide represented the active portion of the molecule. Activity by the peptide would have made synthesis of DBP-maf considerably easier. It showed limited activity in the in vitro assays, however. Future in vivo studies will investigate the ability of DBP-maf to deliver vitamin D to tumor sites and inhibit angiogenesis and tumor growth.

15. SUBJECT TERMS
angiogenesis, prostate, vitamin D, DBP-maf

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	10	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Table of Contents

Introduction

The funding for this project was interrupted when the PI transferred institutions in 2004. Funding was not reinstated until December of 2005, thus this report covers the period from December of 2005 through April of 2006. We have continued to make progress. As stated in our proposal the aims of were to:

- establish the direct effects of individual and combination therapy of DBP-maf and vitamin D in vitro using endothelial and prostate cancer cells. (months 1-9)

- to test each drug in vivo, individually and in combination, to establish optimal doses and treatment schedules. (months 10-30)

- to characterize the pharmacologic profile of each of the drugs and measure targeting of both drugs to the tumor bed. (months 10-36)

The goal of the project is to use DBP-maf, which appears to target to tumor cells and has shown to be anti-angiogenic, and vitamin D in combination therapy of prostate cancer. Vitamin D binds to DBP-maf, therefore it represents a potential stategy to indirectly target vitamin D to tumor sites without causing the hypercalcemia that high systemic levels of vitamin D are known to cause. Both the tumor cells and the supporting blood vessels would be inhibited from growing using the two therapies.

Body

Our initial aim is to characterize the in vitro activity of DBP-maf and vitamin D on endothelial cells as well as tumor cells. We first tested DBP-maf with LnCaP cells in order to determine whether DBP-maf had an effect on tumor cell proliferation. As shown in Figure 1, we observed an ~ 40% reduction in the proliferation of LnCaP cells with DBP-maf. Vitamin D showed ~ 60% reduction but there was no observed additive or synergistic effect when the drugs were combined.
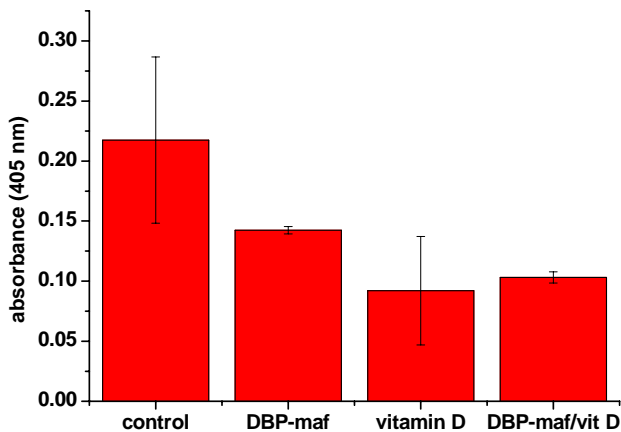

Figure 1. DBP-maf inhibits proliferation of LnCaP cells. LnCaP cells were plated at 1,000 cells/well in 24 well plates and incubated overnight at 37° C in Medium 199 (5% FBS). Medium was changed to serum free medium and dbp-maf (5 ug/ml) and/or vitamin D (0.1 nM) was added. Cells were incubated for 72 hours and quantitated using an acid phosphatase colorimetric assay. Results are plotted +/- s.d.
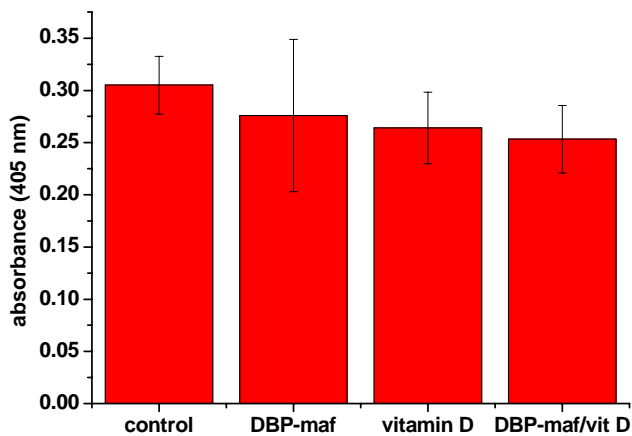
Figure 2. DBP-maf and vitamin D show weak inhibition of proliferation in LnCaP Ln3 metastatic cells. Ln3 cells were plated at a density of 2,500 cells/well in 24 well plates. Cells were incubated overnight in Medium 199 (5% FBS) at 37° C. Medium was changed to serum-free Medium 199. DBP-maf (5 ug/ml) and/or vitamin D (.01 nM) were added and cells were incubated for 72 hours then quantitated using an acid phosphatase colorimetric assay. Results are plotted +/- s.d.

We then repeated the experiment using the LnCaP Ln3 metastatic cells (Figure 2). Interestingly, the metastatic cells showed little response to the same doses of drugs that inhibited the parental line.

Proliferation studies were then performed to test the ability of DBP-maf to inhibit human endothelial cells. Human umbilical vein endothelial cells (HUVEC) were stimulated to proliferate using serum (5% fetal bovine serum) and fibroblast growth factor (FGF-2) a known stimulator of endothelial cell proliferation and migration. As shown in Figure 3, DBP-maf showed weak inhibition of proliferation at 25 ug/ml. At lower concentrations there was no observable inhibition. This is in contrast to the inhibition of bovine endothelial cells reported initially (1) and to a recent report by another group using human cells (2). We have found that endothelial cells are extremely sensitive to endotoxin, however, and even a picomolar amount in a protein preparation could account for inhibitory activity. This is a possible explanation for the more potent inhibitory activity seen in these earlier studies. Also, unlike these earlier studies, there was an effect on tumor cell proliferation by DBP-maf. It is not known whether the prostate cell lines are more sensitive to maf or whether they possess receptors for maf that the pancreatic tumor lines did not. There has not as yet been a receptor identified for DBP-maf, although the thrombospondin receptor has been suggested as a possible site (3).
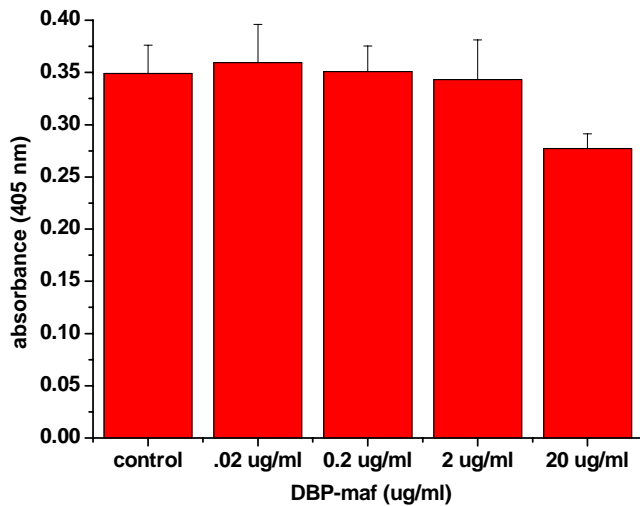
Figure 3. HUVEC proliferation assay using DBP-maf combined with low level vitamin D. HUVECs were incubated overnight (15,000 cells/well) in Medium 199(10% FBS). Medium was changed to 5% FBS with 1 ng/ml FGF-2 and incubated for 72 hours with vitamin D (0.1 nM) and DBP-maf. Cells were quantitated using an acid phosphatase colorimetric assay. Results are +/- s.d.

Cell migration is an important step in the growth of new blood vessels. Using a modified Boyden chamber, the ability of DBP-maf to inhibit endothelial cell migration was tested. As shown in Figure 4, DBP-maf at 5 ug/ml inhibited migration by ~ 25% and with the addition of vitamin D inhibited migration by ~ 40%.
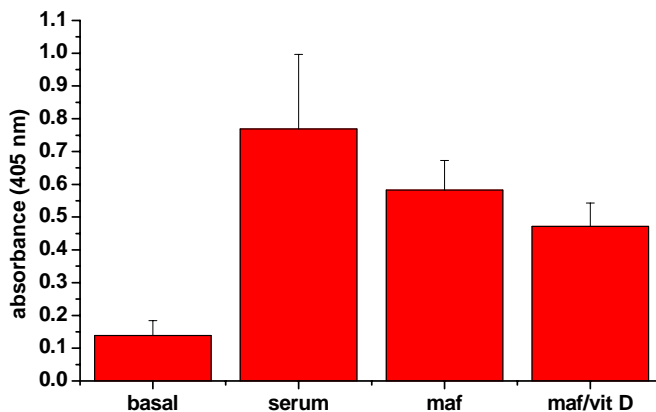

Figure 4. DBP-maf and vitamin D inhibit endothelial cell migration. Human umbilical vein endothelial cells (150,000/well) were added to the top wells of a modified Boyden chamber +/- DBP-maf (5 ug/ml) with or without vitamin D (.01 nM). Fetal bovine serum(10%) was added to the bottom wells and cells were incubated for 6 hours at 37° C. Cells that did not migrate were removed and migrating cells were quantitated using an acid phosphatase colorimetric assay. Results are plotted +/- s.d.

Work by Schneider et al (4) had shown that a 14 amino acid peptide of DBP has activity in bone formation and suggested that this peptide represented the active sequence of DBP-maf that our selective deglycosylation of the native protein exposes. Because the potential use of a peptide would

make the synthesis of DBP-maf a great deal easier, we began initial characterization of this peptide to determine if it could potentially be used in place of the full length molecule in our studies.
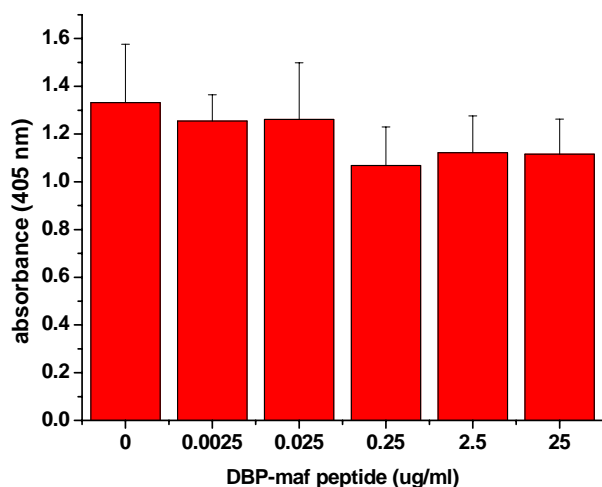

Figure 5. Effect of DBP-maf peptide on LnCaP proliferation.
LnCaP cells (5,000) were plated overnight in 24 well plates. DBP-maf peptide was then added (0% serum) and cells were incubated for 72 hours. Cell numbers were quantitated using an acid phosphatase colorimetric assay. Results are +/- s.d.

Proliferation assays using the peptide showed no significant effect on the proliferation of parental LnCaP cells (Figure 5). Assays using the LnCaP metastatic cells also showed no significant effect on proliferation by the peptide (Figure 6).
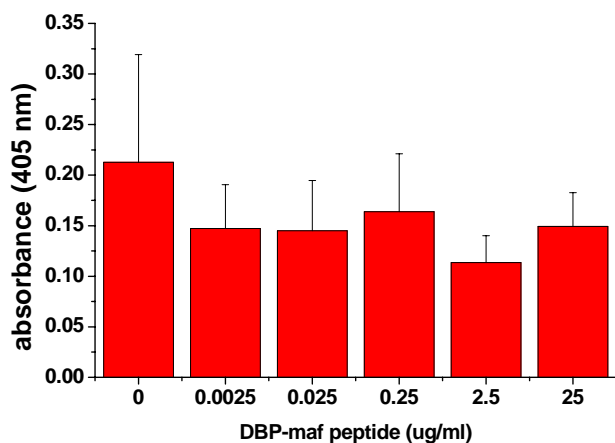

Figure 6. Effect of DBP-maf peptide on metastatic LnCaP proliferation. Cells were incubated overnight then peptide was added. Cells were incubated for 72 hours and quantitated using an acid phosphatase colorimetric assay. results are +/- s.d.

Migration studies were performed to determine whether the peptide had an effect on tumor cell migration. As shown in Figure 7, there was significant inhibition of LnCaP Ln3 cell migration with the peptide, exhibiting a possible "U" shaped dose response curve.
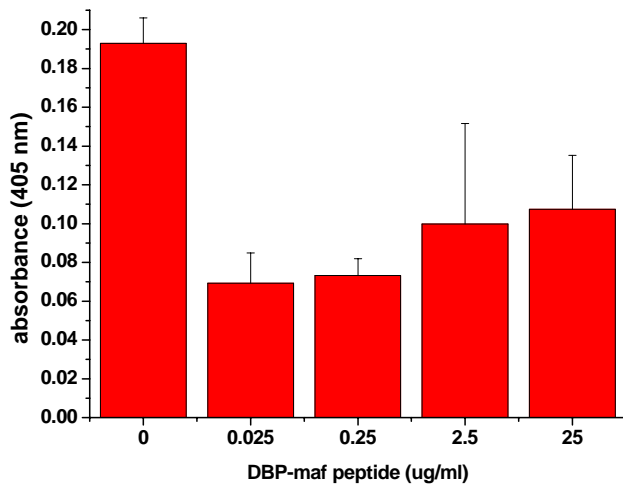
Figure 7. Effect of DBP-maf peptide on migration of LnCaP metastatic cells. Cells (150,000)were plated in the upper wells of a modified Boyden chamber +/- peptide. 10% FBS was added to the the bottom chambers and cells were incubated for 6 hours at 37° C. Cells that did not migrate were wiped from the membrane and migrating cells were quantitated using an acid phosphatase colorimetric assay. Results are +/- s.d.

The assay was repeated using vitamin D in conjunction with the DBP-maf peptide. The addition of vitamin D alone showed inhibition and increased inhibition was observed with the combination of vitamin D and the maf peptide (Figure 8).
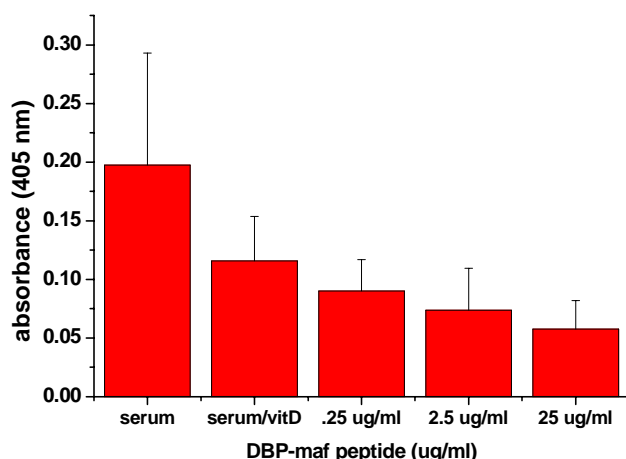
Figure 8. Effect of vitamin D and DBP-maf peptide on LnCaP metastatic cell migration. Cells (150,000)were plated in the upper wells of a modified Boyden chamber +/- peptide with vitamin D (0.1 nM). 10% FBS was added to the the bottom chambers and cells were incubated for 6 hours at 37° C. Cells that did not migrate were wiped from the membrane and migrating cells were quantitated using an acid phosphatase colorimetric assay. Results are +/- s.d.

Although there is some evidence that the DBP-maf peptide has activity in the inhibition of migration, there is so far no data that would suggest that there is any binding affinity between the peptide and vitamin D. This would be important in the strategy that we initially proposed which used DBP-maf not only as a therapy in and of itself, but also as a carrier of vitamin D to enable effective doses of the vitamin to be targeted to tumor sites without having to use the high systemic concentrations that cause hypercalcemia.

Key Research Accomplishments
The key elements that have been discovered so far in the project have been to characterize the in vitro activity of the DBP-maf peptide and full length protein. These assays will be key indicators to develop initial conditions for the in vivo tumor models. Although there are no mechanisms that have been shown to be specifically responsible for DBP-maf activity we are beginning to demonstrate activity based on these in vitro assays that will likely explain its method of action. For example, the inhibition of tumor cell migration shown in Figures 7 and 8 may be important in the inhibition of tumor metastasis.

Reportable Outcomes
Since the project is very early in its inception due to delayed transfer of the grant to a new institution, there are no publications yet resulting from this study, however the data we have accumulated is now being assembled for publication and we anticipate more in the future. We have made, and continue to make progress in characterizing the protein. This will be important in developing an optimal treatment strategy, and in determining the potential of creating synthetic drugs that would likely be easier to manufacture.

Conclusions

We have shown that DBP-maf has direct effects on tumor cells in addition to its already reported activity on endothelial cells. This activity has so far been demonstrated in the inhibition of tumor cell migration, a crucial element of tumor metastasis. We will continue to explore the mechanisms responsible for this inhibition as we begin the in vivo studies using the two parental prostate tumor lines and the two metastatic lines derived from them.

References

1. Kisker, O., Onizuka, S., Banyard, J., Komiyama, T., Becker, C. M., Achilles, E. G., Barnes, C. M., O'Reilly, M. S., Folkman, J., and Pirie-Shepherd, S. R. Generation of multiple angiogenesis inhibitors by human pancreatic cancer. Cancer Res, *61:* 7298-7304, 2001.
2. Kalkunte, S., Brard, L., Granai, C. O., and Swamy, N. Inhibition of angiogenesis by vitamin D-binding protein: Characterization of anti-endothelial activity of DBP-maf. Angiogenesis, *8:* 349-360, 2005.
3. Kanda, S., Mochizuki, Y., Miyata, Y., Kanetake, H., and Yamamoto, N. Effects of vitamin D(3)-binding protein-derived macrophage activating factor (GcMAF) on angiogenesis. J Natl Cancer Inst, *94:* 1311-1319, 2002.
4. Schneider, G. B., Grecco, K. J., Safadi, F. F., and Popoff, S. N. The anabolic effects of vitamin D-binding protein-macrophage activating factor (DBP-MAF) and a novel small peptide on bone. Crit Rev Eukaryot Gene Expr, *13:* 277-284, 2003.

Appendices

None.